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Introduction

Background and Rationale:

Although local therapy is initially efficacious, up to 50% of prostate cancer (CaP) patients develop recurrent disease (1). Androgen deprivation therapy (ADT) in the neo-adjuvant setting is utilized primarily to reduce tumor burden, decreased tumor hypoxia, and increased apoptosis of the tumor Additionally, ADT prompts leukocytes to migrate into the regressing tumors The migration of CD4+ T cells may be beneficial for tumor progression (2-4). If those CD4+ T cells are of the Th1 subset and can directly lyse tumor cells or provide help to cytolytic CD8+ T cells. The cytokine milieu within the tumor microenvironment plays an important role in dictating the tumor response. Ammirante et al. identified tumor infiltrating B cells as key mediators of androgen resistance and tumor progression through production of the lymphotoxin alpha in a mouse model of prostate cancer (5).

Tumor infiltrating B cells have been directly implicated in promoting CaP progression by the production of inflammatory cytokines (6). An early intervention that improves the response to androgen deprivation may be key in preventing CaP progression. A study in colon cancer indicated that depletion of B cells augmented anti-tumor responses and could suppress metastasis (7). However, this is likely tumor type specific as other researchers have found that the absence of B cells impacts the reactivity of CD4+ and CD8+ T cells to tumors (8). Better understanding of the deleterious role of B cells and the possible efficacy of B cell depletion at the time of castration warrants further study for the treatment of prostate cancer.

Patient Humoral Responses and Associated Outcomes:

Investigators at the Deeley Research Centre have previously shown that standard treatments for prostate cancer, in particular ADT and radiation therapy (RT), trigger tumor-specific immune responses as measured by the presence of antibodies that recognize self-antigens (autoantibodies). Specifically, pre- and post-treatment serum from 73 prostate cancer patients was evaluated by Western blot and SEREX (serological identification of antigens by recombinant cDNA expression cloning) antigen arrays for the appearance of treatment-associated autoantibody responses(9). We observed the emergence of autoantibody responses in patients undergoing neoadjuvant ADT (7 of 24, 29.2%), EBRT (5 of 29, 17.2%) and brachytherapy (5 of 20, 25%). In contrast, responses were not seen in patients undergoing surgery (0 of 14) or watchful waiting (0 of 9) (9). Therefore, the tumor specific immune responses were only observed in patients that received ADT and RT.

The clinical status of these patients has now been followed past 5 years (median 73 months). We found that patients who developed autoantibody responses to tumor antigens were more likely to experience biochemical failure (Figure 1, p=0.025, HR=5.99, 1.25-28.75 95% CI). The detection of treatment-induced autoantibodies (made by B cells) suggests that a CD4+ T cell response is also elicited by treatment. CD4+ T cells, termed helper cells, assist B cell and cytolytic CD8+ T cell function. This help is essential for the long-term function and maintenance of CD8+ T cells, which are able to directly kill tumor cells (10). Thus, CD4+ T cells play an essential role in both humoral and adaptive immunity, but are frequently overlooked in immunotherapies in favour of eliciting a response from tumor-specific CD8+ T cell responses.

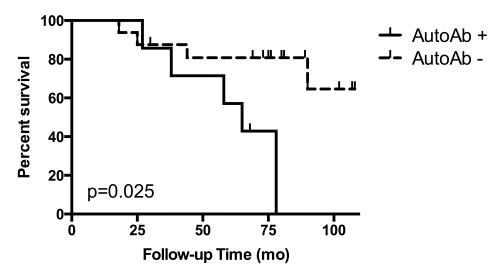


Figure 1. Prostate cancer recurrence in patients treated with ADT and External Beam Radiation Therapy that did (AutoAb+) or did not (AutoAb-) develop treatment induced autoantibody responses

Autoantibody Responses in the Androgen Dependent Shionogi Tumor Model

Interestingly, studies in an androgen-dependent mouse model has revealed that castration (the experimental equivalent of ADT) induces autoantibody responses to an antigen identified as PABPN1 in about 50% of mice. Unexpectedly, mice that developed autoantibody responses to PABPN1 had a shorter latency and higher rate of tumor recurrence, indicating that castration-induced immune responses are associated with inferior outcomes in this model (11). Preliminary experiments in the androgen-dependent Shionogi tumor model indicate that depletion of CD4+ T cells at the time of castration prolongs the time to tumor recurrence (Figure 1). In these mice, the rate of initial tumor regression is similar regardless of treatment group. Mice that received a CD4 did not show anti-body responses against PABPN1. These experiments suggest that altering that modulating the immune

response, by depletion of immune cells, could enhance the effectiveness of ADT and potentially decrease the incidence of castration resistant disease.

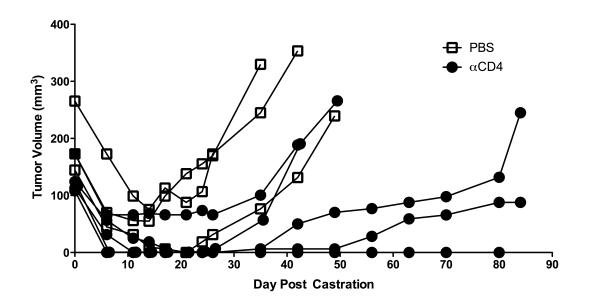


Figure 2. Shionogi tumor bearing mice received either PBS or α CD4 (clone GK1.5) at the time of castration (Day 0) and then weekly maintenance doses. Closed circles indicate individual mice that received α CD4 (n=5) and open squares indicate that receive PBS as a control (n=3).

Key Research Accomplishments

TRAMP-C2 tumors were implanted into C57BL/6 mice and allowed to establish for 30 days prior to surgical castration. Three days prior to castration, mice were injected with anti-CD20 antibody (5D2) to deplete B cells, so that depletion was complete at the time of castration (Appendix I, Figure 1). Control mice received PBS.

CD8+ T cells are Increased in Tumors of Mice that are B cell Depleted

Tumor infiltrating lymphocytes (Figure 3, left panel) were assessed for distribution of CD4 and CD8 T cells (Figure 3), and although PBS treated mice trended towards increased infiltration, it was not statistically significant. There was also a trend towards and increased ratio of CD4+ T cells to CD8+ T cells infiltrating the tumor in PBS treated mice, but this was also not statistically significant (Figure 3, middle panel). Interestingly, there was a significant increase in the percentage of CD8+ T cells in the TIL of CD20-depleted tumors (Figure 3, right panel).

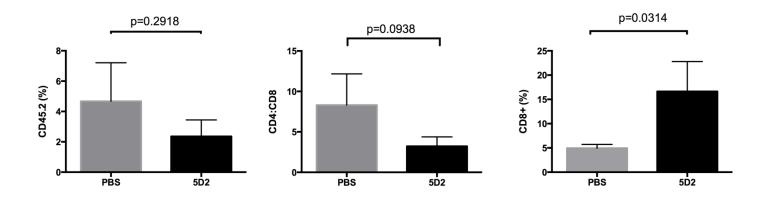


Figure 3. Infiltration of T cells Into TRAMP-C2 tumors. Tumor infiltrating hematopoietic cells were identified by CD45.2 expression (left panel). The ratio of CD4:CD8 T cells was determined by gating on CD45.2+ cells within the tumor. The percentage of CD8+ T cells was determined after gating on CD45.2+ cells. There was a significant increase in the percentage of CD8+ T cells within the tumors of CD20 depleted mice (right panel).

T cells from CD20 depleted mice are more responsive to TCR stimulation

To determine if T cells were functionally affected through the T cell receptor by B cell depletion, lymphocytes were harvested from the tumor draining lymph nodes of castrated tumor bearing mice that had received PBS or 5D2. The cells were CFSE labeled and stimulated with α CD3 for 5 days. In both groups, CD4+ and CD8+ T cells underwent division, however, the 5D2 cells were able to undergo more division and have a higher percentage of fully diluted CFSE cells in comparison to the PBS treated mice.

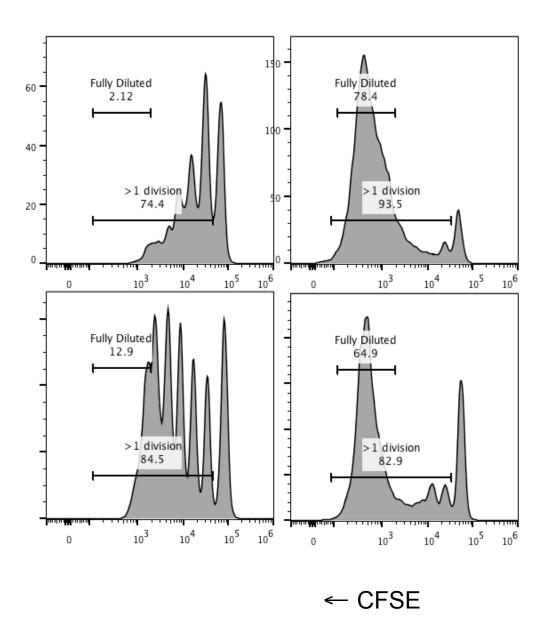


Figure 4. CFSE dilution of CD4+ (top panel) CD8+ (bottom panel)T cells from the TDLN of PBS treated (left panel) or 5D2 treated mice (right panel).

Survival of CD20 Depleted Mice

Although there was an increase in cytolytic T cells into the tumor post CD20 depletion, there was no survival benefit (Figure 5). Due to unforeseen limitations on accessing radiation facilities, we were not able to perform radiation on these mice.

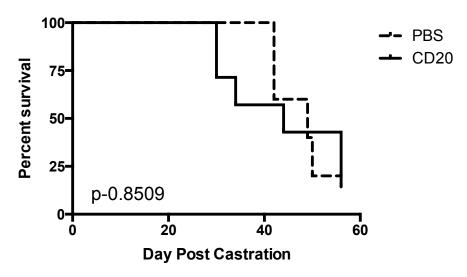


Figure 5. Survival of mice receiving α CD20 at the time of castration.

Reportable Outcomes

Although not a part of the SOW, the analysis of the patient autoantibody responses was recently accepted at the journal "Oncoimmunology" as a study titled "Tumor-associated autoantibodies correlate with poor outcomes in prostate cancer patients treated with androgen deprivation and external beam radiation therapy (Appendix II). This patient data further emphasizes the relevance of studying B cell populations in prostate cancer patients. This DoD award was acknowledged.

During the funding period, an undergraduate student, Gordon Russell, was a co-op student and received a BC Cancer Studentship to work on aspects related to this project.

Conclusion

The role of the B cell was initially thought to be the production of antibodies in response to antigenic stimulation. We (Figure 1), and others, have shown that antibody responses to tumor antigens correlate with tumor progression (12). Additionally, B cells can produce suppressive cytokines including IL-4, IL-10, and TGF- β that can play a role in blunting the ability of T cells to respond to tumors. In murine cancer models, B-cell depletion has had a variety of responses either reducing, enhancing, or having no effect on tumor control. In two studies, in a chemically induced skin cancer model and the other in an implantable breast tumor model, depletion of CD20+ cells removed regulatory B cells, which could be immunosuppressive (13, 14). Interestingly, a group recently studied the effect of B cell depletion using the same α CD20 antibody in a breast cancer model, a cancer that is also affected by hormonal regulation (15). In this case, B cell depletion through α CD20 resulted in the enrichment of B regulatory cells that express low levels of CD20.

These results indicate that CD20 depletion increases the migration of CD8+ T cells into the tumor (Figure 3). Additionally, CD8+ are able to undergo increased proliferation in the absence of CD20+ cells. (Figure 4). However, no additional survival benefit was observed (Figure 5). The increase in cytolytic T cells and improved responsiveness of CD4+ and CD8+ T cells in the tumor draining lymph nodes suggests that B cell depletion as a pre-conditioning regimen for radiation therapy specifically in prostate cancer may be therapeutically beneficial.

In the last 2 years, there has been a tremendous leap forward in the field of immuno-oncology, particularly with the advances in the clinic made with checkpoint inhibitors such as anti-CTLA-4 and anti-bodies that modulate the PD-1 pathway. In both pre-clinical and clinical models, modulation of the immune system in concert with radiation has led to dramatic observations of the abscopal effect (16, 17). In moving forward with B cell depletion, an ability to mediate sufficient response that could generate an abscopal effect will be the necessary.

References

- M. Bolla, G. Van Tienhoven, P. Warde, J. B. Dubois, R. O. Mirimanoff, G. Storme, J. Bernier, A. Kuten, C. Sternberg, I. Billiet, J. L. Torecilla, R. Pfeffer, C. L. Cutajar, T. Van der Kwast, L. Collette, External irradiation with or without long-term androgen suppression for prostate cancer with high metastatic risk: 10-year results of an EORTC randomised study. *Lancet Oncol* 11, 1066-1073 (2010); published online EpubNov (10.1016/S1470-2045(10)70223-0).
- 2. P. Muranski, N. P. Restifo, Adoptive immunotherapy of cancer using CD4(+) T cells. *Current opinion in immunology* **21**, 200-208 (2009); published online EpubApr (10.1016/j.coi.2009.02.004).
- 3. D. M. Pardoll, S. L. Topalian, The role of CD4+ T cell responses in antitumor immunity. *Current opinion in immunology* **10**, 588-594 (1998); published online EpubOct (
- 4. A. Perez-Diez, N. T. Joncker, K. Choi, W. F. Chan, C. C. Anderson, O. Lantz, P. Matzinger, CD4 cells can be more efficient at tumor rejection than CD8 cells. *Blood* **109**, 5346-5354 (2007); published online EpubJun 15 (10.1182/blood-2006-10-051318).
- M. Ammirante, J. L. Luo, S. Grivennikov, S. Nedospasov, M. Karin, B-cell-derived lymphotoxin promotes castration-resistant prostate cancer. *Nature* 464, 302-305 (2010); published online EpubMar 11 (10.1038/nature08782).
- 6. M. Ammirante, J.-L. Luo, S. Grivennikov, S. Nedospasov, M. Karin, B-cell-derived lymphotoxin promotes castration-resistant prostate cancer. *Nature* **464**, 302-305 (2010); published online Epub2010 (
- 7. E. Barbera-Guillem, M. B. Nelson, B. Barr, J. K. Nyhus, K. F. May Jr, L. Feng, J. W. Sampsel, B lymphocyte pathology in human colorectal cancer. Experimental and clinical therapeutic effects of partial B cell depletion. *Cancer Immunology, Immunotherapy* **48**, 541-549 (2000).
- 8. D. J. DiLillo, K. Yanaba, T. F. Tedder, B Cells Are Required for Optimal CD4+ and CD8+ T Cell Tumor Immunity: Therapeutic B Cell Depletion Enhances B16 Melanoma Growth in Mice. *The Journal of Immunology* **184**, 4006-4016; published online EpubApril 1, 2010 (10.4049/jimmunol.0903009).
- 9. N. J. Nesslinger, R. A. Sahota, B. Stone, K. Johnson, N. Chima, C. King, D. Rasmussen, D. Bishop, P. S. Rennie, M. Gleave, P. Blood, H. Pai, C. Ludgate, B. H. Nelson, Standard treatments induce antigen-specific immune responses in prostate cancer. *Clin Cancer Res* **13**, 1493-1502 (2007); published online EpubMar 1 (10.1158/1078-0432.CCR-06-1772).
- 10. J. C. Sun, M. J. Bevan, Defective CD8 T cell memory following acute infection without CD4 T cell help. *Science (New York, N.Y* **300**, 339-342 (2003); published online EpubApr 11 (
- 11. S. Hahn, N. J. Nesslinger, R. J. Drapala, M. Bowden, P. S. Rennie, H. H. Pai, C. Ludgate, B. H. Nelson, Castration induces autoantibody and T cell responses that correlate with inferior outcomes in an androgen-dependent murine tumor model. *International Journal of Cancer* **125**, 2871-2878 (2009).
- 12. C. Domschke, F. Schuetz, Y. Ge, T. Seibel, C. Falk, B. Brors, I. Vlodavsky, N. Sommerfeldt, H. P. Sinn, M. C. Kuhnle, A. Schneeweiss, A. Scharf, C. Sohn, V. Schirrmacher, G. Moldenhauer, F. Momburg, P. Beckhove, Intratumoral cytokines and tumor cell biology determine spontaneous breast cancer-specific immune responses and their correlation to prognosis. *Cancer research* 69, 8420-8428 (2009); published online EpubNov 1 (10.1158/0008-5472.CAN-09-1627).
- 13. P. B. Olkhanud, B. Damdinsuren, M. Bodogai, R. E. Gress, R. Sen, K. Wejksza, E. Malchinkhuu, R. P. Wersto, A. Biragyn, Tumor-evoked regulatory B cells promote breast cancer metastasis by converting resting CD4(+) T cells to T-regulatory cells. *Cancer research* 71, 3505-3515 (2011); published online EpubMay 15 (10.1158/0008-5472.CAN-10-4316).
- T. Schioppa, R. Moore, R. G. Thompson, E. C. Rosser, H. Kulbe, S. Nedospasov, C. Mauri, L. M. Coussens, F. R. Balkwill, B regulatory cells and the tumor-promoting actions of TNF-alpha during squamous carcinogenesis. *Proceedings of the National Academy of Sciences of the*

- *United States of America* **108**, 10662-10667 (2011); published online EpubJun 28 (10.1073/pnas.1100994108).
- M. Bodogai, C. Lee Chang, K. Wejksza, J. Lai, M. Merino, R. P. Wersto, R. E. Gress, A. C. Chan, C. Hesdorffer, A. Biragyn, Anti-CD20 antibody promotes cancer escape via enrichment of tumor-evoked regulatory B cells expressing low levels of CD20 and CD137L. *Cancer research* 73, 2127-2138 (2013); published online EpubApr 1 (10.1158/0008-5472.CAN-12-4184).
- 16. E. F. Stamell, J. D. Wolchok, S. Gnjatic, N. Y. Lee, I. Brownell, The abscopal effect associated with a systemic anti-melanoma immune response. *International journal of radiation oncology, biology, physics* **85**, 293-295 (2013); published online EpubFeb 1 (10.1016/j.ijrobp.2012.03.017).
- 17. L. Deng, H. Liang, B. Burnette, M. Beckett, T. Darga, R. R. Weichselbaum, Y. X. Fu, Irradiation and anti-PD-L1 treatment synergistically promote antitumor immunity in mice. *The Journal of clinical investigation*, (2014); published online EpubJan 2 (10.1172/JCl67313).

Appendix I

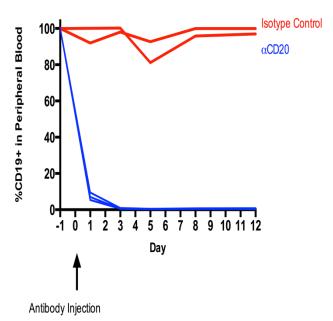


Figure 1. Levels of B cells (CD19+) in the peripheral blood of mice after injection of anti-CD20.

Tumor-associated autoantibodies correlate with poor outcomes in prostate cancer patients treated with androgen deprivation and external beam radiation therapy.

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Running Title: Tumor antibodies indicate CaP recurrence

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Summary: Prostate cancer patients frequently receive radiation therapy, with or without androgen deprivation therapy. We show that treatment-associated immune responses correlate with poor outcome in patients receiving androgen deprivation and external beam radiation therapy. Thus, current treatments for prostate cancer induce detrimental immune responses in some patients. Our findings suggest that immune modulation may be warranted during hormone and radiation therapy to divert tumor immunity away from autoantibody production toward more beneficial cytolytic responses.

Summary: Prostate cancer patients frequently receive radiation therapy, with or without androgen deprivation therapy. We show that treatment-associated immune responses correlate with poor outcome in patients receiving androgen deprivation and external beam radiation therapy. Thus, current treatments for prostate cancer induce detrimental immune responses in some patients. Our findings suggest that immune modulation may be warranted during hormone and radiation therapy to divert tumor immunity away from autoantibodies toward more beneficial cytolytic responses.

ABSTRACT

Purpose: It is becoming increasingly clear that many standard cancer treatments trigger anti-tumor immune responses that influence tumor control. The nature and magnitude of these responses vary depending on the type of tumor and treatment modality. In prostate cancer, we have previously reported that radiation and androgen deprivation therapy (ADT) induce tumor-associated autoantibody responses. In this study, we assessed the relationship between autoantibody responses and clinical outcome. Non-metastatic prostate cancer patients (n=23) received treatment with neoadjuvant & concurrent androgen deprivation therapy (ADT) and external beam radiation therapy (EBRT).

Methods and Materials: Patient sera were collected before, during, and up to 1 year following the completion of treatment. Western blots were used to detect treatment-associated autoantibodies reactive to human prostate cancer cell line lysates. Primary outcome was biochemical recurrence using the consensus-derived Phoenix definition with a median follow-up period of 73 months.

Results: Treatment-associated autoantibodies were detected in 30% of patients treated with ADT and EBRT. Five-year biochemical failure free survival (BFFS) was 78% for all patients. Patients that developed autoantibody responses to tumor antigens had a 57% 5-year BFFS, whereas this increased to 81% in patients that did not develop an autoantibody response.

Conclusions: Overall, the presence of tumor reactive autoantibodies was associated with higher risk of biochemical failure (p=0.025, HR=5.99, 1.25-28.75 95% CI).

INTRODUCTION

Prostate cancer is the most common cancer in North American men. For patients with clinically localized disease, the primary treatment options are surgery or radiation therapy in the form of external beam radiation (EBRT) or brachytherapy (BT). In the case of high-risk disease (e.g. clinical stage ≥T3, Gleason score ≥8 or prostate specific antigen (PSA) >20), patients are typically offered radiation therapy in combination with androgen deprivation therapy (ADT) ^{1, 2}. In these patients, treatment with ADT and EBRT is associated with biochemical failure (BF) rates of 20% at 5-years increasing to 50% at 10-years ^{3, 4}. Thus, improved treatments for high-risk prostate cancer are required.

Intriguingly, ADT and EBRT appear to work synergistically to improve patient outcomes, whereas ADT does not improve outcomes when combined with radical prostatectomy ⁵. ADT is thought to increase tumor cytoreduction and apoptosis while decreasing tumor hypoxia, which may enhance the tumoricidal effects of radiation ⁶. ADT has immunological effects, such as promoting thymopoiesis, which, in turn, may facilitate infiltration of T cells into the prostate to exert anti-tumor effects ^{7, 8}. Radiation treatment can improve the ability of the immune system to recognize tumors by several mechanisms including increased expression of the death receptor Fas and enhanced antigen presentation through upregulation of Major Histocompatibility Complex (MHC) class I on tumors ⁹. In addition, there is increasing evidence that the response to radiation treatment involves a mechanism that depends on Toll-Like Receptor 4 recognition of necrotic tumor cells, a form of immunogenic cell death ¹⁰. This may, in part, also explain the abscopal effect, in which tumor regression occurs outside the radiation field ¹¹. Although the mechanisms underlying the abscopal effect have yet to be determined, it has been proposed that inflammation and tumor death at the radiation site lead to anti-tumor immune responses that can spread to non-irradiated tumors. The

combination of immunogenic tumor cell death, inflammation, and enhanced antigen presentation at the tumor site provides the priming and activation signals that are necessary for sustained T cell mediated anti-tumor responses. As a result, ADT and EBRT may act synergistically to activate immune responses against prostate cancer ^{5, 6}. Therefore, the concept of treatment-induced immune responses remains an important research topic that merits further investigation.

A central component of the humoral immune response is antibody production by B cells, which is dependent on Th2 cytokines produced by CD4 T cells. Studies have shown that tumor-specific humoral immune responses are induced by ADT, radiation, or vaccine approaches in some prostate cancer patients ¹²⁻¹⁴. We have previously reported that the incidence of tumor-associated autoantibodies in patients receiving ADT and EBRT was increased in comparison to those receiving other treatment modalities, such as brachytherapy or radical prostatectomy ¹⁵. As the development of new seroreactivities would suggest antigen spread, we anticipated that treatment-induced autoantibodies would correlate with a lack of biochemical failure. In this study, we assessed the relationship between treatment-associated autoantibody responses and biochemical failure in a cohort of 23 patients receiving ADT and EBRT with more than 5-years median follow-up. Unexpectedly, our study indicates that treatment-associated autoantibody responses may be associated with unfavourable patient outcomes. While striking, it underscores the need to further understand the important interaction between standard treatments and anti-cancer immunity particularly as we move into an era where active immunotherapies are being deployed in the clinic.

SUBJECTS AND METHODS

Subjects. This study received approval by the Research Ethics Board of the British Columbia Cancer Agency and University of British Columbia. Fifty-six patients with non-metastatic prostate cancer who had elected to receive androgen deprivation and external beam radiation therapy with curative intent were recruited at the British Columbia Cancer Agency - Vancouver Island Centre in

 Victoria, British Columbia between December 2003 to May 2006. The majority of patients (16) had high-risk disease (any of the following: stage≥T3a, Gleason Score=8-10 PSA >20), 6 patients had intermediate (stage T2b-T2c or Gleason Score=7 or PSA 10-20), and one patient had low-risk disease but received ADT and EBRT due to bulky disease. Some patients were excluded from further analysis because they had received ADT prior to the first blood collection or were lost to follow-up. Of 56 patients recruited, 23 met the inclusion criteria for this study (Table 1).

Patients received neoadjuvant and concomitant ADT as single agent or combination regimens at the discretion of the treating physicians. Regimens were typically a combination of a luteinizing hormone-releasing agonist and an anti-androgen. Neoadjuvant ADT was typically given for one-year prior and concurrently with EBRT. EBRT was planned and treated with 3D conformal EBRT techniques, using computerized tomography simulation, multi-field beam arrangement to target the prostate gland and seminal vesicles. The prescribed dose of EBRT was 74 Gy delivered in 2 Gy daily fractions over a period of 7.5 weeks.

Blood Collection. Blood samples were collected with informed written consent from study participants. Serum was collected at the first visit, and subsequent serum samples were collected at approximately 3-month intervals during treatment, followed by 6-month intervals for the first year after treatment and timed to coincide with clinical assessment of PSA. Blood was collected in serum separator tubes, centrifuged at 2500 rpm for 10 min, aliquoted and stored at -80°C. PSA values were obtained from patient records. Biochemical failure was defined as nadir +2 ng/ml as per the Phoenix definition ¹⁶.

Detection of autoantibodies by immunoblotting. Western blots were performed as previously described ¹⁵. The human prostate cancer cell line LNCaP was obtained from the American Type Culture Collection. Briefly, 400 μg of protein isolated from LNCaP cells was separated on a single

lane by standard SDS-PAGE and transferred to nitrocellulose. Sera were diluted 1:500 in Blotto (5% dry milk powder, 0.1% Tween 20, 50 mmol/L Tris, 150 mmol/L NaCl) and incubated for 1 hour at room temperature using the Mini Protean II MultiScreen multichannel immunoblotting device (Bio-Rad, Mississauga, ON). Membranes were incubated for 1 hour at room temperature with horseradish peroxidase-conjugated goat anti-human IgG secondary antibody (1:10,000; Heavy and Light chains; Jackson ImmunoResearch) and visualized by enhanced chemiluminescence. All serum samples were assessed by immunoblot at least twice. Autoantibody responses were considered positive if they developed within one-year of completing EBRT.

Detection of actin by immunoblotting. Nitrocellulose membranes were rehydrated in water for 10 minutes. To remove the primary human sera and secondary IgG, blots were incubated for 10 minutes at room temperature with gentle shaking in Restore[™] Western Blot Stripping Buffer (Thermo Scientific). Blots were then washed thoroughly (5 times for 5 minutes at room temperature) in TBST. A 5% milk solution was used to block and blots were reprobed with mouse anti-actin (Sigma #A2228) at a 1:50,000 dilution overnight at 4°C with gentle shaking. The next day, blots were washed 5 times for 5 minutes at room temperature in TBST followed by incubation with anti-mouse IgG IR800 secondary antibody (Rockland #610-132-121; 1:20,000). After 1 hour in secondary antibody, blots were washed and imaged using Li-Cor Odyssey Imager.

Statistical analysis. Log-rank tests were performed to determine significance for biochemical failure in autoantibody positive and negative patients. Risk, which incorporates stage, Gleason score, and PSA level, and the development of autoantibdodies was analyzed by Cox regression analysis.

RESULTS

 Detection of tumor-associated autoantibodies. To assess treatment-associated immune responses, patient sera were immunoblotted against whole cell lysates from the human prostate cancer cell

line LNCaP as previously described ¹⁵. All results were confirmed by at least two independent immunoblots. Sera were scored as positive if they showed the appearance of one or more bands by Western blot during or within one year of treatment. Seven of the twenty-three (30.4%) patients developed one or more seroreactivities. Of the sixteen high-risk patients, 6 (37.5%) developed autoantibody responses and one (16%) of the intermediate risk patients did as well. The low risk patient that was treated with ADT and EBRT did not develop an autoantibody response. In four of these patients (57.1 %), new seroreactivity was observed after ADT but before EBRT. Figure 1 shows the Western blot results for two healthy donor controls (Fig. 1A), one representative patient who did not develop autoantibodies (Fig. 1B; Patient 052), and two representative patients (Fig. 1C; Patient 054, Fig 1D; Patient 170; arrows) who scored positive for treatment-associated autoantibodies.

Autoantibody responses are associated with poor prognosis following ADT and EBRT. The median follow-up time (the time from the end of treatment to the last available PSA value or death) was 73 months (range 18 – 108 months). Nine patients (39.1%) experienced BF. Of the seven patients who were autoantibody positive, five (71.4%) experienced BF. By Kaplan-Meier analysis, patients who developed autoantibody responses during treatment showed a significantly higher rate of BF (Fig. 1C; p=0.025, HR=5.99, 1.25-28.75 95% CI). Multivariate analysis was performed with risk group and autoantibody status (Table 2). This analysis indicated that neither risk group (p= 0.704, HR 1.342, 0.294-6.126) nor the development of autoantibodies (p=0.058, HR 4.283, 0.954-19.224) is a significant independent predictor of BF (Table 2), likely due to the small number of patients in this study cohort.

DISCUSSION

Tumor-associated autoantibodies are detectable in patients with a variety of cancers including prostate cancer and may have prognostic value ¹⁷. The current study was undertaken to investigate whether standard therapies could, a) result in tumor-associated humoral responses and b) whether these changes correlate with treatment outcomes in patients receiving ADT and EBRT. For high risk and some intermediate risk disease, the combination of ADT and EBRT is a standard treatment option but is associated with biochemical failure > 20% at 5 years for patients with more aggressive disease ¹⁸. Of the patients treated with ADT and EBRT, 30.4% developed an autoantibody response during or within one year of treatment. Of the autoantibody positive patients, 71.4% experienced BF compared to only 25% of patients without autoantibody responses.

These results are consistent with our laboratory findings in the Shionogi tumor model where the detection of autoantibodies specific for poly(A) binding protein correlated with tumor recurrence after androgen deprivation by castration ¹⁹. In another study evaluating autoantibody responses in patients receiving a poxvirus-based vaccine and EBRT, autoantibody responses had no impact on survival ¹³. However, when stratified by certain antigen-specific autoantibodies, a trend towards worse outcomes was observed ¹³. The specificity of the antigen responses may be essential to determine whether or not autoantibody responses are beneficial or detrimental. Smith and colleagues noted that autoantibody responses to specific tumor antigens varied between individuals as well as type of treatment ¹². We found that the majority of autoantibody responses coincide with BF, suggesting that this type of the immune response might promote disease recurrence. However, these conclusions will require validation on a larger cohort of patients, as well as further investigation to uncover the underlying mechanism of these autoantibody responses.

The increased rate of BF in patients with tumor-associated autoantibodies indicates that B cell activation may promote tumor aggressiveness. It is possible that the combination of ADT with EBRT promotes a tumor microenvironment where stimulation of prostate-resident B cells results in chronic production of inflammatory cytokines that leads to tumor progression ²¹. Consistent with this alternative hypothesis is a report demonstrating that autoantibody production in the K14-HPV16 model of squamous carcinogenesis recruits and regulates tumor associated macrophages and mononuclear phagocytes through interaction of IgG with Fcy receptors suggesting that the detection of autoantibodies in the serum of patients may represent an immunosuppressive tumor environment ²². Moreover, a recent finding by Karin and colleagues showed that tumor infiltrating B cells can directly promote the development of androgen-independent prostate cancer progression via the production of pro-inflammatory cytokines, specifically lymphotoxin- α^{23} . In another study of colon cancer, depletion of B cells augmented anti-tumor responses and suppressed metastasis ²⁴. Taken together, these data suggest that B cells may contribute to tumor pathogenesis in prostate cancer and that immune modulation through B cell depletion or blockade of inflammatory cytokines produced by B cells could be therapeutically beneficial in a subset of prostate cancer patients that receive ADT + EBRT.

Patients receiving ADT + EBRT represent those with the highest risk for recurrence, and within this patient population we have identified a sub-group of patients with a deleterious immune response that has a higher risk of recurrence. Randomized clinical trials have demonstrated that the addition of ADT to EBRT results in enhancing rather than compromising prostate cancer control and survival, but is still associated with high rates of biochemical failure ². Understanding the immunological profile of those patients with unfavourable prognosis may help to stratify patients that would benefit most from additional treatments, such as B cell depletion. Longitudinal monitoring of our prostate cancer patients is needed to shed light on the interactions amongst B and T cells and their respective contributions to treatment-associated immune responses.

Preventing autoantibody development and inducing a more beneficial Th1 response may prove to be important directions for treatment of high-risk prostate cancer patients. Such investigations will pave the way for the development of immunomodulating agents that improve the effectiveness of standard therapies for this disease.

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REFERENCES

- 1. Alexander AS, Mydin A, Jones SO, Christie J, Lim JT, Truong PT, Ludgate CM. Extremerisk prostate adenocarcinoma presenting with prostate-specific antigen (PSA)>40 ng/ml: prognostic significance of the preradiation PSA nadir. Int J Radiat Oncol Biol Phys 2011;81:e713-9.
- 2. Ludgate CM, Lim JT, Wilson AG, Alexander AS, Wilson KS. Neoadjuvant hormone therapy and external beam radiation for localized prostate cancer: Vancouver Island Cancer Centre experience. Can J Urol 2000;7:937-43.
- 3. Tendulkar RD, Reddy CA, Stephans KL, Ciezki JP, Klein EA, Mahadevan A, Kupelian PA. Redefining High-Risk Prostate Cancer Based on Distant Metastases and Mortality After High-Dose Radiotherapy With Androgen Deprivation Therapy. Int J Radiat Oncol Biol Phys 2012;82:1397-404.
- 4. Bolla M, Van Tienhoven G, Warde P, Dubois JB, Mirimanoff RO, Storme G, Bernier J, Kuten A, Sternberg C, Billiet I, Torecilla JL, Pfeffer R, et al. External irradiation with or without long-term androgen suppression for prostate cancer with high metastatic risk: 10-year results of an EORTC randomised study. Lancet Oncol 2010;11:1066-73.
- 5. Sorrentino C, Musiani P, Pompa P, Cipollone G, Di Carlo E. Androgen deprivation boosts prostatic infiltration of cytotoxic and regulatory T lymphocytes and has no effect on disease-free survival in prostate cancer patients. Clin Cancer Res 2011;17:1571-81.
- 6. Hayden AJ, Catton C, Pickles T. Radiation therapy in prostate cancer: a risk-adapted strategy. Curr Oncol 2010;17 Suppl 2:S18-24.
- 7. Mercader M, Bodner BK, Moser MT, Kwon PS, Park ESY, Manecke RG, Ellis TM, Wojcik EM, Yang D, Flanigan RC, Waters WB, Kast WM, et al. T cell infiltration of the prostate induced by androgen withdrawal in patients with prostate cancer. Proceedings of the National Academy of Sciences of the United States of America 2001;98:14565-70.

- 8. Drake CG. Prostate cancer as a model for tumour immunotherapy. Nature reviews. Immunology 2010;10:580-93.
- 9. Chakraborty M, Abrams SI, Camphausen K, Liu K, Scott T, Coleman CN, Hodge JW. Irradiation of tumor cells up-regulates Fas and enhances CTL lytic activity and CTL adoptive immunotherapy. J Immunol 2003;170:6338-47.
- 10. Apetoh L, Ghiringhelli F, Tesniere A, Criollo A, Ortiz C, Lidereau R, Mariette C, Chaput N, Mira JP, Delaloge S, Andre F, Tursz T, et al. The interaction between HMGB1 and TLR4 dictates the outcome of anticancer chemotherapy and radiotherapy. Immunological reviews 2007;220:47-59.
- 11. Ludgate CM. Optimizing Cancer Treatments to Induce an Acute Immune Response: Radiation Abscopal Effects, PAMPs, and DAMPs. Clinical Cancer Research 2012;18:4522-5.
- 12. Smith HA, Maricque BB, Eberhardt J, Petersen B, Gulley JL, Schlom J, McNeel DG. IgG responses to tissue-associated antigens as biomarkers of immunological treatment efficacy. J Biomed Biotechnol 2011;2011:454861.
- 13. Nesslinger NJ, Ng A, Tsang K-Y, Ferrara T, Schlom J, Gulley JL, Nelson BH. A Viral Vaccine Encoding Prostate-Specific Antigen Induces Antigen Spreading to a Common Set of Self-Proteins in Prostate Cancer Patients. Clinical Cancer Research 2010;16:4046-56.
- 14. Dunphy EJ, McNeel DG. Antigen-specific IgG elicited in subjects with prostate cancer treated with flt3 ligand. J Immunother 2005;28:268-75.
- 15. Nesslinger NJ, Sahota RA, Stone B, Johnson K, Chima N, King C, Rasmussen D, Bishop D, Rennie PS, Gleave M, Blood P, Pai H, et al. Standard Treatments Induce Antigen-Specific Immune Responses in Prostate Cancer. Clinical Cancer Research 2007;13:1493-502.
- 16. Roach M, 3rd, Hanks G, Thames H, Jr., Schellhammer P, Shipley WU, Sokol GH, Sandler H. Defining biochemical failure following radiotherapy with or without hormonal therapy in men with clinically localized prostate cancer: recommendations of the RTOG-ASTRO Phoenix Consensus Conference. Int J Radiat Oncol Biol Phys 2006;65:965-74.

- 17. Stockert E, Jager E, Chen YT, Scanlan MJ, Gout I, Karbach J, Arand M, Knuth A, Old LJ. A survey of the humoral immune response of cancer patients to a panel of human tumor antigens. J Exp Med 1998;187:1349-54.
- 18. Pickles T, Keyes M, Morris WJ. Brachytherapy or conformal external radiotherapy for prostate cancer: a single-institution matched-pair analysis. Int J Radiat Oncol Biol Phys 2010;76:43-9.
- 19. Hahn S, Nesslinger NJ, Drapala RJ, Bowden M, Rennie PS, Pai HH, Ludgate C, Nelson BH. Castration induces autoantibody and T cell responses that correlate with inferior outcomes in an androgen-dependent murine tumor model. International Journal of Cancer 2009;125:2871-8.
- 20. Aragon-Ching JB, Williams KM, Gulley JL. Impact of androgen-deprivation therapy on the immune system: implications for combination therapy of prostate cancer. Front Biosci 2007;12:4957-71.
- 21. de Visser KE, Korets LV, Coussens LM. De novo carcinogenesis promoted by chronic inflammation is B lymphocyte dependent. Cancer cell 2005;7:411-23.
- 22. Andreu P, Johansson M, Affara NI, Pucci F, Tan T, Junankar S, Korets L, Lam J, Tawfik D, DeNardo DG, Naldini L, de Visser KE, et al. FcRgamma activation regulates inflammation-associated squamous carcinogenesis. Cancer cell 2010;17:121-34.
- 23. Ammirante M, Luo J-L, Grivennikov S, Nedospasov S, Karin M. B-cell-derived lymphotoxin promotes castration-resistant prostate cancer. Nature 2010;464:302-5.
- 24. Barbera-Guillem E, Nelson MB, Barr B, Nyhus JK, May KF, Jr., Feng L, Sampsel JW. B lymphocyte pathology in human colorectal cancer. Experimental and clinical therapeutic effects of partial B cell depletion. Cancer Immunol Immunother 2000;48:541-9.

Figure 1. Treatment-associated responses to prostate tumor antigens. Western blot analysis of serum

4from 2 healthy donor controls and 3 patients probed against LNCaP cell lysate. The timing of the

5 sample collection for each patient is indicated. New seroreactivities are indicated with an arrow. (A)

9Two health donor controls showed no seroreactivity, (B) patient 052 who did not develop an

10 12 autoantibody response throughout treatment, (C) patient 054 who was treated with ADT + EBRT

13 4 developed a new response 8 months post EBRT and (D) patient 170, who developed two new

15 16 responses 1 month post EBRT. Each blot was re-probed against actin without the multichannel device to

18 19 ensure equal protein loading across each lane. The lines indicate the original slot blot lane for each

20 2 Isample.

²⁶Figure 2. Treatment-associated autoantibody responses are associated with increased likelihood of control of control

Table 1. Subject Characteristics				
Number of Subjects	23			
Age, median (range)	69.7 (51.3-81.2)			
Gleason Score, n (%) 6 7 8-10	7 (30.4) 11 (47.9) 5 (21.7)			
Stage, n (%) T1 T2 T3	8 (34.8) 12 (52.2) 3 (13.0)			
PSA at diagnosis Median (ng/ml) (range)	11.0 (3.1-100)			
Risk group, n (%) Low Intermediate High	1 (4.3) 6 (26.1) 16 (69.6)			

Table 2. Cox-multivariate analysis					
Variable	Hazard Ratio	95% CI	p-value		
Risk Group	1.342	0.294-6.126	0.704		
Autoantibody Status	4.283	0.954-19.224	0.058		

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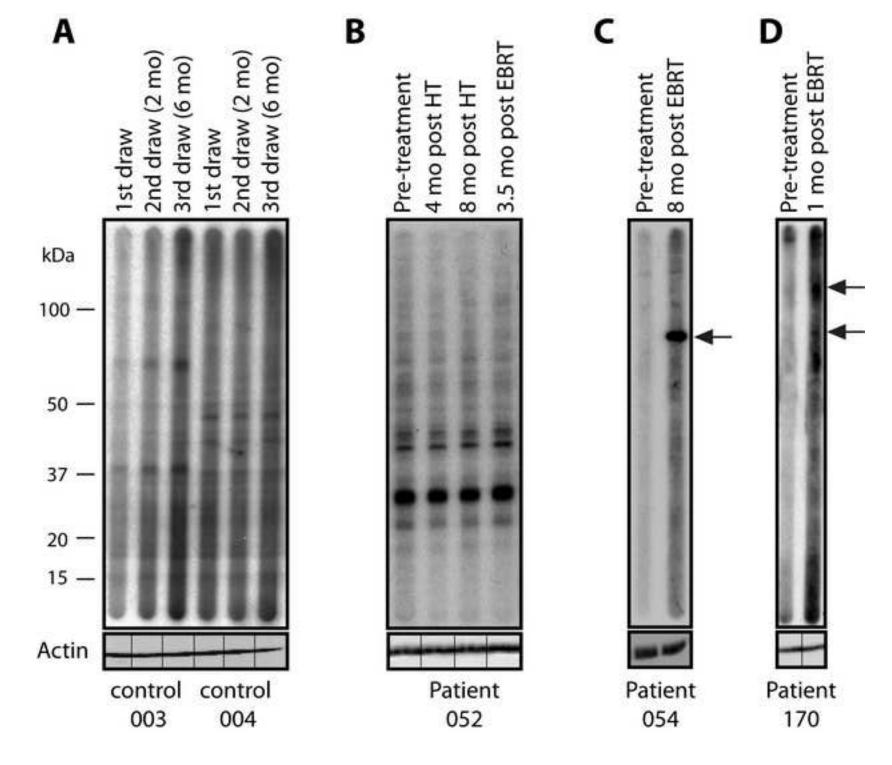


Figure 2
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